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Short Communication

Development of single and multispecies detection methods for the surveillance and monitoring of marine pests in New Zealand

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Editor's note:
This special issue of *Aquatic Invasions* includes papers from the 17th International Conference on Aquatic Invasive Species held in San Diego, California, USA, on August 29 to September 2, 2010. This conference has provided a venue for the exchange of information on various aspects of aquatic invasive species since its inception in 1990. The conference continues to provide an opportunity for dialog between academia, industry and environmental regulators within North America and from abroad.

Abstract

New Zealand's current surveillance programme for marine pests does not include methods for identification of organisms in the water column, therefore dispersive forms go undetected. Molecular methods provide an opportunity to detect dispersive forms thereby contributing to a more robust surveillance programme. Additionally, New Zealand has become a signatory to the IMO Ballast Water Convention and there is scope for developing enumeration methods for detection of viable organisms as well as species-specific molecular probes for indicator bacteria. We describe here the outcome of experiments testing the effects of various matrices (e.g. sediment, biofilms, benthic assemblage, seawater) against a quantitative PCR (QPCR) assay developed for *Potamocorbula amurensis*. The limit of detection of the assay for sediments and benthic assemblages ranged between one to five larvae per 10 g wet weight. We also report a QPCR assay for *Vibrio cholerae*, one of the indicator species in the Ballast Water Convention. Quantitative PCR of seawater spiked with different concentrations of culture showed that assay sensitivity was insufficient to detect the regulatory limit of 1 colony forming unit (CFU) per 100 mL. More recent work suggests that sufficient sensitivity can be achieved with cultivation based methods (most probable number-QPCR [MPN-QPCR]). We are currently appraising several multispecies detection methods including micro-arrays and next generation sequencing for their suitability in marine pest detection. The advantages and disadvantages of each are discussed herein. We highlight the need to consider issues relating to sampling design when using molecular methods for surveillance and compliance monitoring.

Key words: molecular, probes, QPCR, micro-arrays, next generation sequencing

Currently in New Zealand, the surveillance of marine pests is conducted biannually at seven ports in a MAF Biosecurity NZ co-ordinated programme. A variety of sampling techniques are used including epibenthic sled tows, diver and drop camera searches (wharf piles, seafloor, etc), and shoreline searches (Inglis et al. 2006). The purpose of the surveillance programme is early detection of new organisms, facilitating cost effective pest management decisions leading to their containment or limiting their spread. To date, marine surveillance programmes in New Zealand do not monitor for dispersive life stages of invasive species in the water column. As a result, incursions of pest organisms in their early life stages could have gone undetected.

New Zealand port surveillance surveys target six unwanted marine pests. These include *Asterias amurensis* Lütken, 1871 (Echinodermata), *Potamocorbula amurensis* Schrenck, 1861 (Mollusca), *Carcinus maenas* Rumph, 1705 (Arthropoda), *Eriocheir sinensis* Milne-Edwards, 1853 (Arthropoda), *Sabella spallanzanii* Viviani, 1805 (Annelida), and *Caulerpa taxifolia* (Vahl 1802) Agardh (Chlorophyta). Recently, we have developed an *in situ* hybridisation assay and a sandwich hybridisation assay for *A. amurensis* (Mountfort et al. 2007; Smith et al. 2011), and in

conjunction with The Commonwealth Scientific and Industrial Organisation (CSIRO), Australia, have optimised a quantitative PCR (QPCR) assay for this species (Bax et al. 2006; Smith et al. 2007). We have also developed a QPCR assay for *P. amurensis* (Smith et al. 2012), and we are presently modifying the QPCR assay developed at the South Australian Research and Development Institute (SARDI) for *S. spallanzanii*. Traditional PCR methods, but not QPCR, have also been developed by other workers for detection of *C. maenas* and *C. taxifolia* (Harvey et al. 2009). All QPCR assays we have developed include a salmon sperm DNA-based internal control as described in Haugland et al. (2005).

A key consideration in the application of molecular methods for surveillance is the sensitivity of the assay for a variety of environmental samples collected using different sampling methods. To investigate this we determined the limit of detection (LoD) of our *P. amurensis* QPCR assay by spiking *P. amurensis* larvae into a variety of samples.

Plankton net (20-µm mesh size) samples were collected from a depth of 19 m in Tasman Bay, New Zealand (S 41.03.48', E173.05.47'). Subsamples (100 mL) were preserved immediately with RNAlater™ (200 mL). Approximately 0.23 m^3 of water was concentrated per sample. Sediment samples were collected from Tasman Bay (S 41.15.46', E 173.12.80') from a depth of 11 m using a sediment grab sampler. Subsamples (10 g) were placed in 50-mL Falcon tubes and preserved immediately with RNAlater™ (40 mL). Nine 20-cm × 20-cm and three 10-cm × 10-cm Perspex settling plates were placed 30 cm below the water's surface at the Port of Nelson, New Zealand (S 41.15.18', E173.16.56') for 3 weeks and 3 months respectively. At the conclusion of the inoculation period, biofilms and benthic assemblages on the plates where scraped into 50-mL Falcon tubes containing RNAlater™ (40 mL).

Water samples were centrifuged and the resulting pellets assayed. Excess RNAlater™ was decanted from sediment, biofilm and benthic assemblage samples. Benthic assemblage samples were homogenized using a hand-held blender (Kenwood, New Zealand) for 60 seconds. A given number of *P. amurensis* larvae (stored in RNAlater™) were transferred using micro-pipettes and an inverted microscope to the first tube of a Mo Bio™ DNA isolation kit; different Mo Bio™ kits were used depending on the type of sample (Table 1). A water sample pellet or the appropriate mass of matrix (Table 1) was then added to this tube. Upon completion of genomic isolation, each sample was spiked with DNA from salmon testes (D1626, Sigma, St. Louis, MO) (15 ng for no matrix, water and 0.25 g samples, and 600 ng for 10 g samples). Positive (reference samples of *P. amurensis* DNA) and negative (DNA extraction control and un-spiked samples) controls were included in subsequent QPCR assays. Results are summarized in Table 1.

All negative controls yielded no QPCR signal. The LoD for all sample matrices was one *P. amurensis* larva, except for the 10 g benthic assemblage where the LoD was five larvae. In future studies, it would be useful to concentrate larger seawater volumes than those considered here. To test sediment samples >10 g, larger scale DNA extraction methods would be required.

We have also developed a QPCR assay to detect indicator bacteria in ballast water. This opportunity arose because of New Zealand's decision to accede to the IMO Ballast Water Convention. A key component of the Convention relates to three groups of indicator bacteria; in each case the regulatory limit is 1 CFU per 100 mL of ballast water. One of these indicator bacteria is *Vibrio cholerae* strains O1 and O139. A QPCR assay was adapted for strain O1 based on previously described methods (Blackstone et al. 2007). To test the suitability of the assay, an overnight culture of *V. cholerae* O1 (5.6×10^8 cells mL^{-1}) was serially diluted (seven 10-fold dilutions) and 1 mL of each dilution was added to 100 mL of seawater and filtered onto 47 mm, 0.4 µm polycarbonate filters (HTTP04700, Millipore, Bedford, MA). The filters were then extracted using a Mo Bio PowerSoil™ DNA isolation kit and 4 µL of the extract analysed by QPCR targeting the CTX gene (Blackstone et al. 2007). The lower limit of the assay was 22 cells per reaction, or 550 cells per 100 mL (Figure 1). Therefore, QPCR sensitivity was insufficient for detection at the regulatory limit of 1 CFU per 100 mL. Recent research initiatives to increase the sensitivity to ≤1 organism per 100 mL of ballast water include: 1) A most probable number-QPCR (MPN-QPCR) assay — samples are serially diluted in alkaline peptone water (APW) and tested by QPCR after overnight incubation; 2) Sample filtration followed by filter incubation on Thiosulfate Citrate Bile Salts (TCBS) agar and QPCR colony screening. Initial

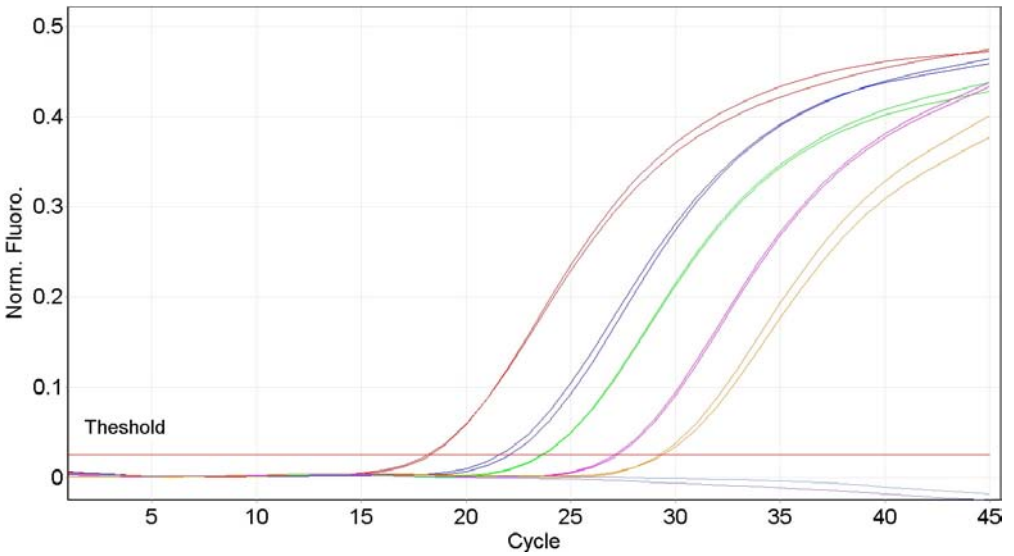

Figure 1. Quantitative PCR profile for a culture of *Vibrio cholerae* strain O1 (5.6 × 108 cells/ml).

Table 1. Quantitative PCR results and DNA isolation kit used for each *Potamocorbula amurensis* larvae spiking experiment. + positive result, - negative result.

Matrix	Number of larvae	DNA Isolation Kit	Number of replicates	Volume/Mass	QPCR	
					Potamocorbula. amurensis	Salmon sperm
No Matrix	1	PowerSoil™	3		+	+
Water	1	PowerSoil™	3	0.23 m^3	+	+
Sediment	1	PowerSoil™	3	0.25 g	+	+
Sediment	1	PowerMax™ Soil	3	10 g	+	+
Biofilm	1	PowerBiofilm™	3	0.25 g	+	+
Benthic assemblage	1	PowerBiofilm™	3	0.25 g	+	+
	1*	PowerMax™ Soil	1	10 g	-	+
Benthic assemblage	3*	PowerMax™ Soil	1	10 g	-	+
	5*	PowerMax™ Soil	3	10 g	+	+

* Samples were diluted 1:10 with Milli-Q water.

Table 2. Summary of performance characteristics of three next generation sequencers as they relate to their potential in pest detection.

Method	Principal	Read length (bp)	Other Features
Ion Torrent PGM	Combines semi-conductor chip and biochemical technologies	100-200	Machine relatively inexpensive (<$US50K)
Roche FLX series (454 sequencing)	Chemiluminescent signal after bead capture of amplified DNA fragments	>600	Up to 10^6 reads per run
Roche GS Junior	As for Roche FLX	~ 600	Up to 10^5 reads per run Rapid sequencing of amplicons/genes

results indicate that sensitivities of 1 CFU per 100 mL can be achieved with these methods (Marek pers. comm.).

Quantitative PCR is sensitive and specific, making it a useful tool for marine biosecurity monitoring. Multiplexing can be used in this assay, enabling the detection of >3 species in a single sample, however, our ultimate goal is to monitor for many (>100) species simultaneously. Two platforms that we have considered to achieve this are micro-arrays and next generation sequencing. Both approaches have advantages

and disadvantages. Micro-array chips can be re-used and give a direct readout of results, but are limited to pre-defined target-species detection. In contrast, organism detection via sequencing is limited only by PCR primer specificity, allowing simultaneous detection of diverse target and non-target species. Sequence data can then be identified using current databases. A disadvantage of sequencing is the required multi-stage sample processing.

In selecting an appropriate sequencer for next-generation sequencing, we have included performance characteristics of some sequencers in Table 2. *In silico* sequence analysis of the 28S rDNA from five marine pests showed that although a 100 bp read length would discriminate among the five target pest species, > 400 bp would be required to separate closely related non-target species from target pests. Of the sequencers considered, the Roche GS Junior pyrosequencing system meets these read length requirements and pilot studies are planned. Among array systems on the market, CustomArray® potentially addresses our specific requirement for an array that detects an a priori defined, relatively small (~ 100) group of target organisms. CustomArray® is also re-usable at least four times. A key feature of CustomArray® is the compact Electrosense reader which is inexpensive compared to those used in other array systems (i.e., optical scanners). Currently we are investigating the potential of this array as a multispecies detection tool using contrived communities of 15 of New Zealand and Australia's invasive marine species

The research initiatives described here detail the performance of QPCR in various sample matrices as well as the transformation of single species detection methods to multispecies methods. Outside the scope of this manuscript is the basis of sampling design that defines the sample number required for detection in surveillance. A theoretical basis for sampling design for port surveillance based on binomial probability of detection has been advanced previously (e.g., Hayes et al. 2005) as well as its application in existing port surveillance in New Zealand (e.g., Inglis et al. 2006). Taking into account sampling costs these studies suggest that application of molecular methods might be effort limited to seawater samples.

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